Clades of Adeno-Associated Viruses Are Widely Disseminated in Human Tissues

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The potential for using *Adeno-associated virus* (AAV) as a vector for human gene therapy has stimulated interest in the *Dependovirus* genus. Serologic data suggest that AAV infections are prevalent in humans, although analyses of viruses and viral sequences from clinical samples are extremely limited. Molecular techniques were used in this study to successfully detect endogenous AAV sequences in 18% of all human tissues screened, with the liver and bone marrow being the most predominant sites. Sequence characterization of rescued AAV DNAs indicated a diverse array of molecular forms which segregate into clades whose members share functional and serologic similarities. One of the most predominant human clades is a hybrid of two previously described AAV serotypes, while another clade was found in humans and several species of nonhuman primates, suggesting a cross-species transmission of this virus. These data provide important information regarding the biology of parvoviruses in humans and their use as gene therapy vectors.

Adeno-associated virus (AAV) is a member of the genus *Dependovirus*, which lies within the *Parvoviridae* family (17). An interest in this family of viruses has been stimulated because of their potential use as gene transfer vectors (14).

Little is known about the biology of AAV infections, although a significant proportion of humans and nonhuman primates have antibodies in their blood that react to some of the six existing serotypes of AAV (5, 7). This suggests that primates are hosts for infection with AAV, although the clinical sequelae of these infections have yet to be identified.

The study of AAV has been limited to the previously described six serotypes, of which five were isolated as contaminants in laboratory preparations of adenoviruses (1, 3, 16). Our lack of understanding of AAV clinical infections has complicated the search for clinical isolates of the virus. Members of our laboratory recently described a strategy for evaluating latent or persistent AAV genomes from tissues of asymptomatic nonhuman primates through the use of PCR. These studies led to the discovery of two novel AAV serotypes, called AAV7 and AAV8, that have improved properties as vectors for gene therapy (10). In nonhuman primates, AAV sequences were quite prevalent and heterogenous (9).

The goal of this study was to determine if latent AAVs exist in humans, and if so, to characterize their structural, serologic, and functional properties.

MATERIALS AND METHODS

Collection of primate tissues. Our sources of nonhuman primate tissues were described previously (9). Human tissues were collected under two independent

IRB protocols approved by the Institutional Review Board of the University of Pennsylvania from either surgical procedures, postmortem examinations, or organ donors through two major national human tissue providers, the Cooperative Human Tissue Network and the National Disease Research Interchange. The human tissues used for this study were comprised of 18 different tissue types that included the colon, liver, lung, spleen, kidney, brain, small bowel, bone marrow, heart, lymph nodes, skeletal muscle, ovary, pancreas, stomach, esophagus, cervix, testes, and prostate. The tissue samples came from a diverse group of individuals of different genders, races (Caucasian, African American, Asian, and Hispanic), and ages (23 to 83 years). Among the 259 samples analyzed from 250 individuals, approximately 28% of the tissues were associated with pathology.

Detection and isolation of AAV sequences. Total cellular DNAs were extracted from human and nonhuman primate tissues as described previously (10). The molecular prevalence and tissue distribution of AAVs in humans were determined by either signature or full-length cap PCRs using primers and conditions that were similar to those used for nonhuman primate analyses (9, 10). The same PCR cloning strategy used for the isolation and characterization of an expanded family of AAVs in nonhuman primates was deployed for the isolation of AAVs from selected human tissues (9, 10). A total of 67 capsid clones isolated from human tissues were characterized (hu.1 to hu.67). From nonhuman primate tissues, 86 cap clones were sequenced, among which 70 clones were from rhesus macaques, 6 clones were from cynomolgus macaques, 3 clones were from pigtailed macaques, 2 clones were from a baboon, and 5 clones were from a chimpanzee.

Computational analysis of primate AAV sequences. From all sequence contigs, VP1 open reading frames were analyzed. VP1 protein sequences were aligned with ClustalX (22) and an in-frame DNA alignment was produced with the BioEdit (11) software package. Phylogenies were inferred with the MEGA v2.1 and TreePuzzle packages. Neighbor-joining, maximum parsimony (18), and maximum likelihood (21) algorithms were used to confirm similar clusterings of sequences in monophylic groups. Clades were then defined from a neighbor-joining phylogenetic tree of all protein sequences. The amino acid distances were estimated by making use of Poisson correction. The statistical robustness of the analysis was estimated by bootstrapping with 1,000 replicates. Sequences were considered monophylic when they had a connecting node within a genetic distance of 0.05. A group of sequences originating from three or more sources was considered a clade. Homoplasy was screened for by implementation of the split decomposition algorithm (2). Splits that were picked up in this manner were then further analyzed for recombination by use of the Bootscan algorithm in the Simplot software (20). A sliding window of 400 nucleotides (nt) (10 nt/step) was used to obtain 100 bootstrap replicate neighbor-joining trees. Subsequently, split decomposition and neighbor-joining phylogenies were inferred from the putative

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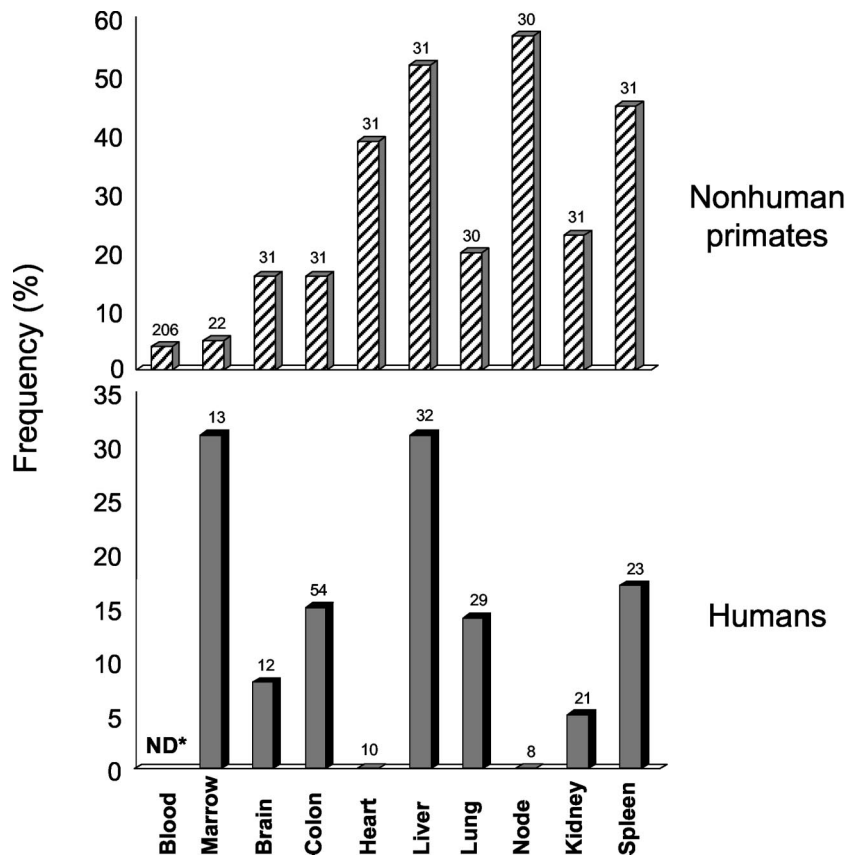† G.G. and L.H.V. contributed equally to this work.

FIG. 1. Tissue distribution of AAV sequences in primate tissues. Total cellular DNAs were extracted from 10 major tissues, including the blood, from nonhuman primates (top) and humans (bottom) and were subjected to PCR analysis for AAV sequences. The x axis indicates the tissues analyzed and the y axis represents the frequencies of detection of AAV sequences. The total number of samples examined for each tissue of human or nonhuman primate origin is listed at the top of each bar.

recombination fragments. A significant improvement of bootstrap values, a reduction of splits, and a regrouping of the hybrid sequences with their parents were considered the criteria for recombination.

Evaluation of primate AAVs as gene transfer vectors. Primate AAV vectors were produced by a previously described transencapsidation method (10). Vectors expressing enhanced green fluorescent protein (EGFP) were used to examine their in vitro transduction efficiencies in 84-31 cells and to study their serological properties. For in vivo studies, human α-antitrypsin (A1AT) was selected as a sensitive and quantitative reporter gene for the vectors and was expressed under the control of a cytomegalovirus-enhanced chicken β-actin promoter. Four to 6-week-old NCR nude mice were treated with novel AAV vectors at a dose of 10^{11} genome copies per animal through intraportal, intratracheal, and intramuscular injections for liver-, lung-, and muscle-directed gene transfer, respectively. Serum samples were collected at different time points after gene transfer and A1AT concentrations were determined by an enzyme-linked immunosorbent assay.

Nucleotide sequence accession numbers. New AAV sequences from the PCR clones presented in this paper have been submitted to GenBank. The accession numbers are AY530553 to AY530629.

RESULTS AND DISCUSSION

Human tissues were obtained from a variety of sources, and DNAs were evaluated for endogenous AAV sequences by PCRs with oligonucleotides specific to homologous regions of the cap gene. Figure 1 summarizes a portion of the results of this screen of 259 human samples of 18 different tissue types derived from 250 individuals. The data were compared to similar studies using an expanded pool of tissues from rhesus

monkeys, cynomolgus and pigtailed macaques, baboons, and chimpanzees. The prevalence of AAV sequences in human and nonhuman primate tissues was similar (19 and 18%, respectively). Livers and spleens were the predominant sites of AAV infection in both human and nonhuman primates, although endogenous sequences were also frequently found in colons and bone marrow from humans and lymph nodes from nonhuman primates. Quantitative PCR studies indicated that endogenous AAV in human tissues is present in low quantities and unlikely to be present as a result of germ line transmission (data not shown).

To better understand the origins and consequences of endogenous AAVs in humans, we attempted to recover and fully sequence full-length cap structures from human tissues; isolates from nonhuman primates, in addition to what was previously described, were also included. A total of 108 new and unique isolates (from 55 human and 53 nonhuman primates) were identified (clones from the same individual with fewer than four amino acid differences were deemed redundant and eliminated from the analysis).

This pool of primate AAV cap sequences was analyzed for phylogenetic relationships by using a variety of computational approaches. Sequences were aligned with the ClustalX1.81 program and phylogenies were assessed by the neighbor-joining, maximum parsimony, and maximum likelihood algorithms
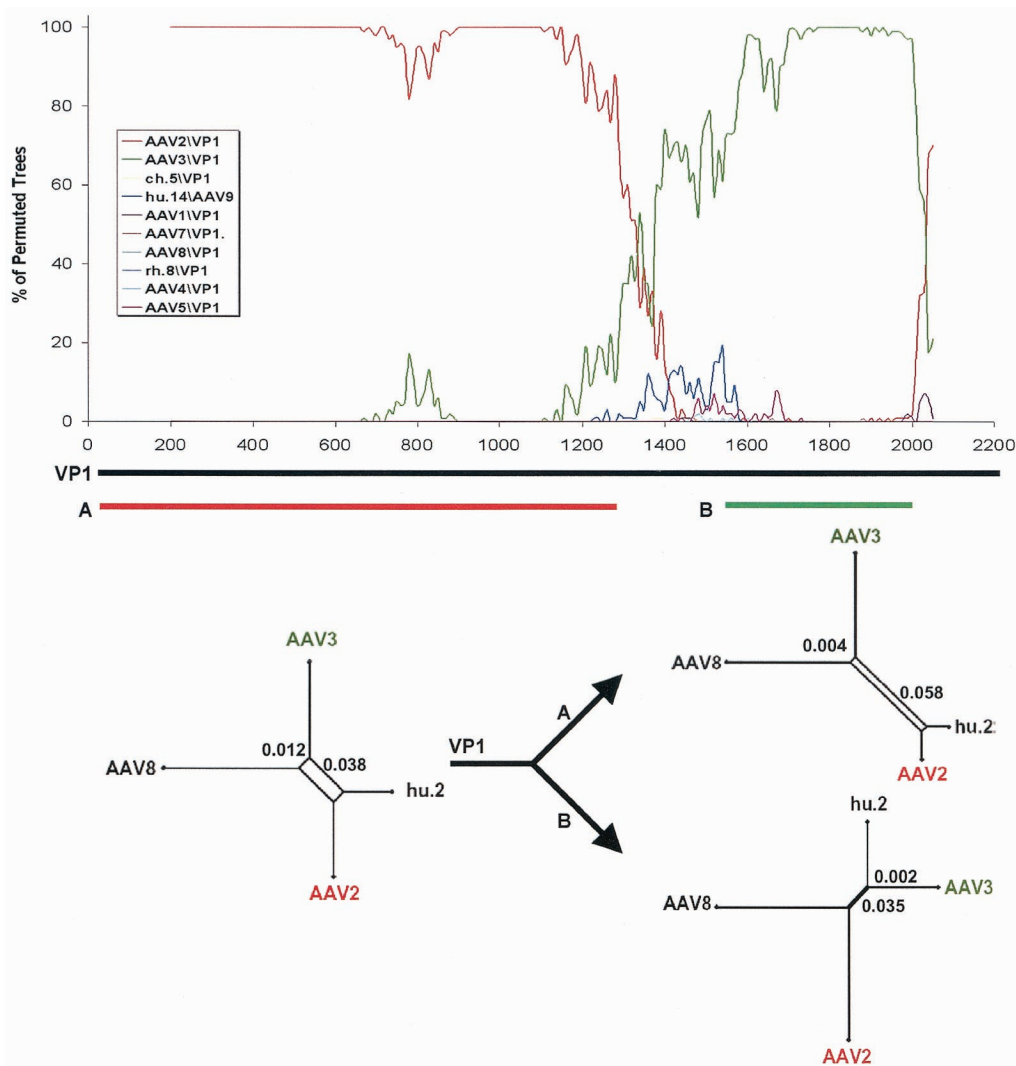
FIG. 2. Recombination analysis of AAV2-AAV3 hybrid clade representative hu.2. (Top) Bootscanning analysis of hu.2 versus representatives of all other clades showing a distinct phylogenetic relatedness between the 5′ (A) and the 3′ (B) segments (indicated below) of the entire VP1 capsid sequence. (Bottom) Split decomposition analysis of AAV2, AAV3, and the AAV2-AAV3 hybrid group representative hu.2, with AAV8 used as an outgroup. The left panel shows the analysis of the entire VP1 sequence, while on the right the conflicting phylogenies are resolved for segments A and B. The hamming distances are indicated on the splits. All branches were supported by a minimum of 85% bootstrap values ($n = 1,000$).

(18, 19, 21). Each method yielded a similar clustering of sequences. The phylogeny of AAV was further evaluated for evidence of recombination through a sequential analysis of split decomposition (2) and bootscanning (20). Split decomposition analysis depicts parallel events in a set of sequences with a tree-like network rather than a bifurcating tree. The bootscanning algorithm then further verifies these putative recombination events by visualizing the mosaic structure of a given sequence. A number of different cap sequences amplified from eight different human subjects showed phylogenetic relationships to AAV2 (5′) and AAV3 (3′) around a common breakpoint at position 1400 of the cap DNA sequence, consistent with recombination and the formation of a hybrid virus (Fig. 2). This is the general region of the cap gene in which recombination was detected in isolates from a mesenteric lymph node of a rhesus macaque (9).

The phylogenetic analyses were repeated, excluding the clones that were positively identified as hybrids. In this analysis, goose and avian AAVs were included as outgroups (6). Figure 3 summarizes a neighbor-joining tree; similar relationships were obtained by maximum parsimony and maximum likelihood analyses. These analyses demonstrated 11 phylogenetic groups, which are summarized in Table 1. When a group contained nonredundant but phylogenetically similar members from three or more sources, it was called a clade; otherwise, it was called a clone or set of clones. The clades were defined as clades A to F, as shown in Table 1. The five categories of clones are as follows: the previously described AAV3 and AAV5 clones that are clearly distinct from one another but were not detected in our screen; a group of three similar clones from a rhesus macaque that are closely related to AAV4; two similar clones, represented by rh.8, from different rhesus macaques
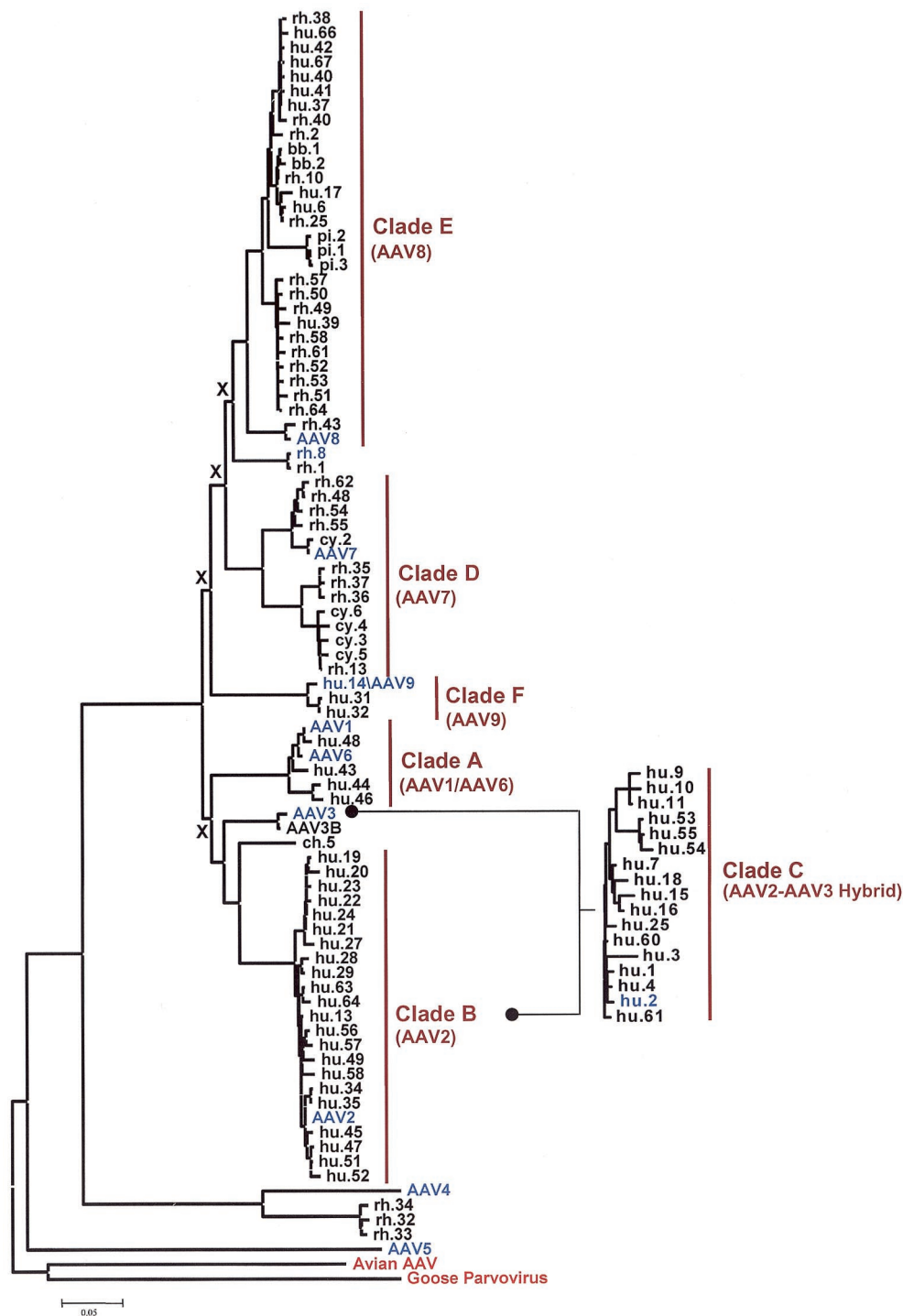
FIG. 3. Neighbor-joining phylogenies of the VP1 protein sequence of primate AAVs. Major nodes with bootstrap values of <75 are indicated with an "X." A goose parvovirus and an avian AAV (6) were used as the outgroup. Clades are indicated by name and by vertical lines to the right of the taxa from which they are made. The nomenclature for the taxa is either the serotype name or a reference to the species source (hu, human; rh, rhesus macaque; cy, cynomolgus macaque; bb, baboon; pi, pigtailed macaque; ch, chimpanzee), followed by a number indicating the order in which they were sequenced. Clade C was identified and positively determined to have originated through the recombination of known clades. The AAV2-AAV3 hybrid clade originated after one recombination event, and its unrooted neighbor-joining phylogeny is shown.

TABLE 1. Classification of number of sources per clade or clone[a]

Clade or clone	Representative members	No. of sequences for species[b]						Reference for published sequence
		Humans	Rhesus macaques	Cynomolgus macaques	Baboons	Chimpanzees	Pigtail macaques	
Clades								
A	AAV1 (AAV6)	3 (4)						13
B	AAV2	12 (22)						1
C	AAV2-AAV3 hybrid	8 (17)						
D	AAV7		5 (10)	5 (5)				10
E	AAV8	7 (9)	7 (16)		1 (2)		1 (3)	10
F	AAV9	3 (3)						
Clones								
AAV3								4
AAV4			1 (3)					16
AAV5								3
ch.5						1 (1)		
rh.8			2 (2)					

[a] The species origins of the six AAV clades and five individual AAV clones (or sets of clones) are represented.
[b] Numbers in parentheses indicate the total numbers of nonredundant sequences isolated.

that are not related to previously described AAV serotypes; and a single unique clone from chimpanzees, called ch.5. The previously described AAV1 and AAV6 clones are members of a single clade for which four isolates were recovered from three humans. The clades representing AAV2 and the AAV2-AAV3 hybrid are the most abundant of those found in humans (22 isolates from 12 individuals for AAV2 and 17 isolates from 8 individuals for the AAV2-AAV3 hybrid). A clade containing AAV7 is unique to rhesus and cynomolgus macaques, with 15 members being isolated from 10 different animals. The clade containing AAV8 is interesting because it is found in both human and nonhuman primates: 9 isolates were recovered from 7 humans and 21 isolates were obtained from 9 different nonhuman primates, including rhesus macaques, a baboon, and a pigtail monkey.

The last clade was derived from isolates from three humans and did not contain a previously described serotype. Polyclonal antisera were generated against a representative member of this clade, and a comprehensive study of serologic cross-reactivity between the previously described serotypes was performed (Table 2). The isolate from this clade was serologically distinct from the other known serotypes and therefore the clade was called the AAV9 clade.

Additional experiments were performed to evaluate the relationship of phylogenetic relatedness to function, as measured by serologic activity and tropism. Polyclonal antisera generated against the nine known serotypes were used to evaluate cross-neutralization (Table 2). For the purposes of discussion, we defined a new serotype as one for which the neutralization titer by heterologous sera was at least 16-fold less than the neutralization titer against the homologous vector in reciprocal titrations. These data confirmed the phylogenetic groupings of the different clones and clades except for an unanticipated serological reactivity of the structurally distinct AAV5 and AAV1 serotypes (the ratios of heterologous to homologous titers were 1/4 and 1/8, respectively, in reciprocal titrations). It should be noted that the previously described AAV1 and AAV6 serotypes do not segregate by either their phylogeny (Fig. 3) or their serology (the ratios of heterologous to homologous titers were 1/2 and 1/4, respectively, in reciprocal titrations).

The biological tropisms of AAVs were studied by generating transencapsidated vectors in which recombinant AAV2 genomes expressing either GFP or the secreted reporter gene A1AT were packaged with capsids derived from the various clones or clades. The vectors were evaluated for their trans-

TABLE 2. Serologic evaluation of novel AAV vectors[a]

Vector used for rabbit immunization	Titer for vector pseudotype								
	AAV2/1	AAV2/2	AAV2/3	AAV2/4	AAV2/5	AAV2/6	AAV2/7	AAV2/8	AAV2/9
AAV2/1	1/163,840	No NAB	No NAB	No NAB	1/40,960	1/40,960	1/40	No NAB	No NAB
AAV2/2	1/80	1/81,920	1/5,120	1/20	No NAB	1/80	1/40	1/40	No NAB
AAV2/3	1/1,280	1/2,560	1/40,960	1/20	1/40	1/2,560	1/1,280	1/1,280	No NAB
AAV2/4	1/20	No NAB	No NAB	1/1,280	1/40	No NAB	No NAB	No NAB	1/40
AAV2/5	1/20,480	No NAB	1/80	No NAB	1/163,840	1/5,120	1/40	No NAB	No NAB
AAV2/6	1/81,920	No NAB	1/640	1/40	1/40	1/327,680	1/40	No NAB	1/40
AAV2/7	1/1,280	1/640	1/1,280	1/20	No NAB	1/1,280	1/163,840	1/5,120	1/80
AAV2/8	1/20	1/1,280	1/1,280	No NAB	1/20	No NAB	1/640	1/327,680	1/2,560
AAV2/9	No NAB	No NAB	No NAB	No NAB	No NAB	No NAB	1/20	1/640	1/20,480

[a] Rabbit polyclonal antibodies against AAV serotypes 1 to 9 were generated. To test neutralizing and cross-neutralizing reactivities of antisera to each serotype of the AAVCMVEGFP vector, we preincubated the vectors with homologous and heterologous antisera and then incubated them with indicator 84-31 cells for 48 to 72 h. The transduction of 84-31 cells by AAVCMVEGFP vectors was assessed under a UV microscope. Neutralization titers were defined as described previously (10). NAB, neutralizing antibody.
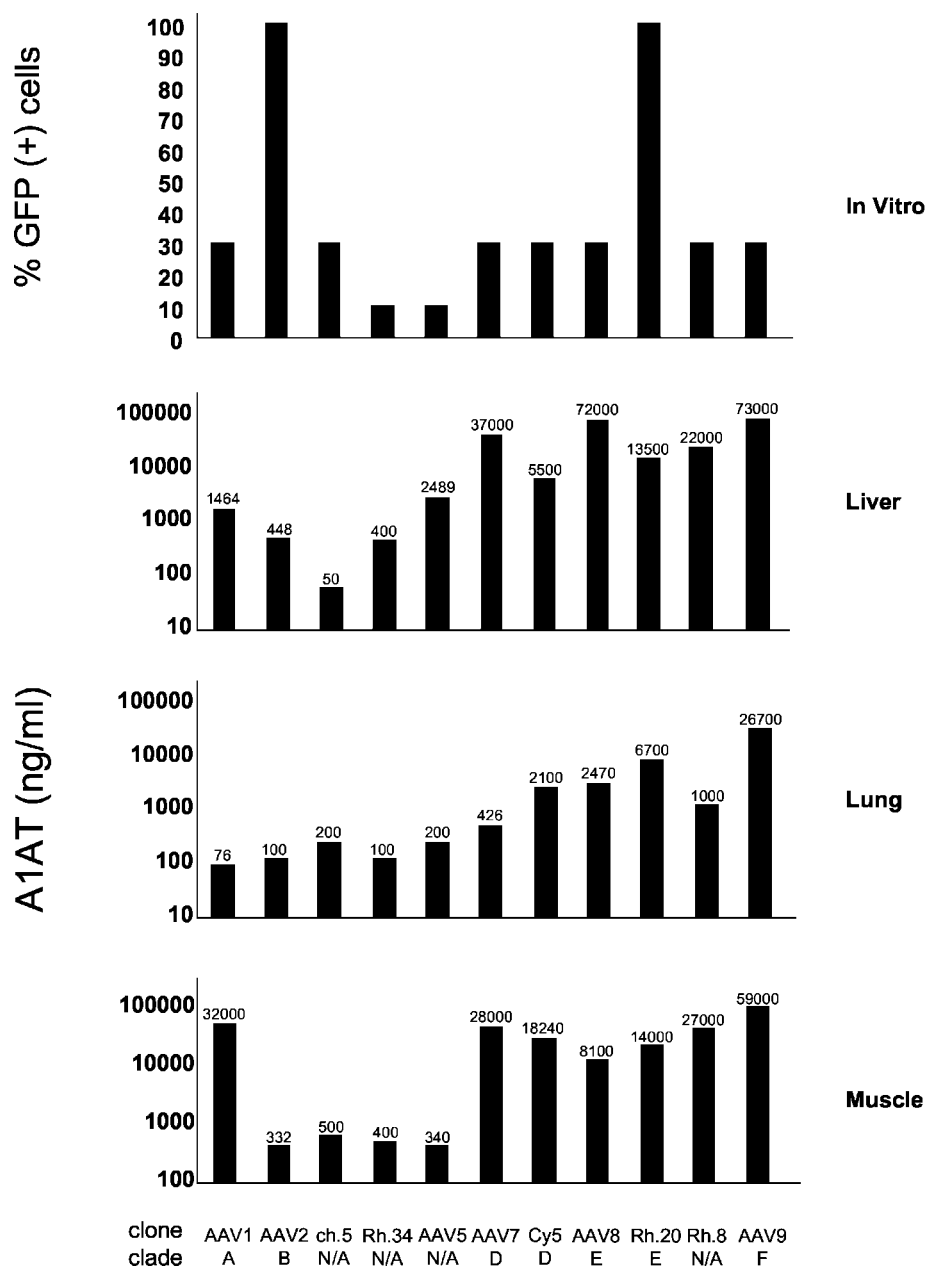
FIG. 4. Evaluation of gene transfer efficiency of novel primate AAV-based vectors in vitro and in vivo. AAV vectors were pseudotyped with capsids as indicated and measured as percentages of GFP^{+} cells. Analyses of in vitro transduction with GFP vectors and in vivo transduction with A1AT vectors (measured as serum A1AT levels, in micrograms per milliliter) were performed as described in Materials and Methods. For in vivo comparisons, the serum A1AT level is indicated at the top of each bar, representing the gene transfer efficiency of each AAV vector.

duction efficiency in vitro, based on GFP transduction, and their transduction efficiency in vivo in the liver, muscle or lung. Vectors expressing EGFP were used to examine their in vitro transduction efficiencies in 84-31 cells and to study their serological properties. For in vivo studies, human A1AT was selected as a sensitive and quantitative reporter gene for the vectors and was expressed under the control of the cytomegalovirus-enhanced chicken β-actin promoter. Four to 6-week-old NCR nude mice were treated with novel AAV vectors at a dose of 10^{11} genome copies per animal through intraportal, intratracheal, and intramuscular injections for liver-, lung-, and

muscle-directed gene transfers, respectively. Serum samples were collected at different time points after the gene transfer, and A1AT concentrations were determined by an enzyme-linked immunosorbent assay. A representative set of assay results is shown in Fig. 4.

In order to compare unique profiles of transduction, we developed a grading system to characterize the relative transduction efficiency of each in vitro and in vivo model (from 0 [lowest] to 3 [highest]). The cumulative functional difference between two vectors with capsids A and B is the sum of the absolute values of differences between the individual assays as

TABLE 3. Functional and structural differences of primate AAVs[a]

Virus	Cumulative difference in gene transfer scores or % cap amino acid differences (in bold)									
	AAV1	AAV2	AAV3	ch.5	AAV4	AAV5	AAV7	AAV8	rh.8	AAV9
AAV1	0	5	ND	4	4	4	2	4	5	4
AAV2	**16.3**	0	ND	3	2	4	7	7	6	9
AAV3	**13.2**	**12.3**	0	ND	ND	ND	ND	ND	ND	ND
Ch.5	**15.5**	**10.5**	**11.5**	0	2	4	6	6	5	8
AAV4	**33.7**	**36.7**	**34.8**	**34.9**	0	2	7	6	5	8
AAV5	**39.1**	**38.8**	**38.5**	**38.4**	**42.7**	0	4	4	3	6
AAV7	**14.1**	**16.7**	**14.9**	**15.6**	**33.2**	**38.5**	0	2	3	2
AAV8	**15.6**	**16.4**	**14**	**15.6**	**33.2**	**38.9**	**11.6**	0	1	2
Rh.8	**14.1**	**15.2**	**14.3**	**14.4**	**33.7**	**39.6**	**12.1**	**8.8**	0	3
AAV9	**17.2**	**17.3**	**15.6**	**14.8**	**34.5**	**39.7**	**17.5**	**14.3**	**12.5**	0

[a] To examine differences in structure and function among primate AAVs, we selected one representative member from each primate AAV clade for comparison. For instance, the data obtained from AAV1 were used to represent clade A, followed by AAV2 for clade B, rh.34 for the AAV4 clade, AAV7 for clade D, AAV8 for clade E, and AAV hu.14 for clade F. AAV5, AAVch.5, and AAVrh.8 stand as single AAV serotypes or genotypes for the comparison. For functional analysis, the in vitro transduction of different AAVCMVEGFP vectors was measured in 84-31 cells that were seeded in a 96-well plate and infected with pseudotyped AAVCMVEGFP vectors at a multiplicity of infection of 10^4 GC per cell. The relative EGFP transduction efficiency was scored as 0, 1, 2, or 3, corresponding to 0 to 10%, 10 to 30%, 30 to 70%, or 70 to 100% green cells, as estimated by using a UV microscope at 48 h postinfection. The in vivo potency of different AAVCBhA1AT vectors was evaluated in NCR nude mice for liver-, lung-, and muscle-directed gene transfer at a dose of 10^{11} GC per animal and was scored as 0, 1, 2, or 3 relative to different serum A1AT levels at day 28 after gene transfer, depending on the route of vector administration (for the liver, 0 = A1AT of <400 ng/ml, 1 = A1AT of 400 to 1,000 ng/ml, 2 = A1AT of 1,000 to 10,000 ng/ml, and 3 = A1AT of >10,000 ng/ml; for the lung, 0 = A1AT of <200 ng/ml, 1 = A1AT of 200 to 1,000 ng/ml, 2 = A1AT of 1,000 to 10,000 ng/ml, and 3 = A1AT of >10,000 ng/ml; for muscle, 0 = A1AT of <100 ng/ml, 1 = A1AT of 100 to 1,000 ng/ml, 2 = A1AT of 1,000 to 10,000 ng/ml, and 3 = A1AT of >10,000 ng/ml). Cumulative differences of the gene transfer scores were calculated as described in the text. ND, not determined. The percentages of difference in cap structure were determined by dividing the number of amino acid differences after a pairwise deletion of gaps by 750, the length of the VP1 protein sequence alignment.

follows: cumulative functional difference = (in vitro A − in vitro B) + (liver A − liver B) + (lung A − lung B) + (muscle A − muscle B). Smaller cumulative functional differences indicate similar profiles with regard to transduction efficiency. Table 3 summarizes the cumulative functional difference scores as well as the % differences in VP1 amino acid sequences in pairwise comparisons.

Unique profiles of biological activity, in terms of the efficiency of gene transfer, were demonstrated for the different clones and clades of AAVs, with substantial concordance between members of a set of clones or a clade (data not shown). This suggests that biological pressures drive the evolution of AAVs.

Our studies point out a number of issues that are relevant to the study of parvoviruses in humans. The prevalence of endogenous AAV sequences in a wide array of human tissues suggests that natural infections with this group of viruses are quite common. The wide tissue distribution of viral sequences and their frequent detection in the liver, spleen, and gut suggest that transmission may occur via the gastrointestinal tract and that viremia may be a feature of the infection. Some earlier reports also documented the detection of AAV sequences in the human female genital tract and suggested that sexual contact could be another route of transmission (8, 23). However, the clinical consequences of infection with AAV have yet to be delineated.

An inspection of the topology of the phylogenetic analysis revealed insight into the relationship between the evolution of the virus and its host restriction. The entire genus *Dependovirus* appears to be derived from avian AAVs, consistent with the work of Lukashov and Goudsmit (15). After the emergence of AAV4 and AAV5, the family diverged into two monophylic groups (Fig. 3), with one containing clades that are specific to humans (clades A, B, and C) and the other comprised of a mixture of clades that were isolated exclusively from humans

(clade F), exclusively from nonhuman primates (clade D), or from both human and nonhuman primates (clade E).

The presence of latent AAVs that are widely disseminated throughout human and nonhuman primates and their apparent predisposition to recombine and to cross species barriers raise important issues. This combination of events has the potential to lead to the emergence of new infectious agents with modified virulence. Assessments of this potential are confounded by the fact that the clinical sequelae of AAV infections in primates have yet to be defined. In addition, the high prevalence of AAV sequences in the liver may contribute to dissemination of the virus in the human population in the setting of allogeneic and xenogeneic liver transplantation. Finally, the finding of endogenous AAVs in humans has implications for the use of AAV for human gene therapy. The fact that wild-type AAV is so prevalent in primates without ever being associated with a malignancy suggests that it is not particularly oncogenic. In fact, the expression of AAV *rep* genes has been shown to suppress transformation (12). A potential complication of AAV gene therapy, however, could be recombination between the vector and endogenous genomes. This could lead to swapping of the inverted terminal repeats, rearrangement in the transgene cassettes, a loss of regulatory elements for regulated gene expression, and other effects.

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